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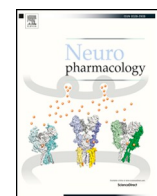
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Ketamine-induced regulation of TrkB-GSK3 β signaling is accompanied by slow EEG oscillations and sedation but is independent of hydroxynorketamine metabolites

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HIGHLIGHTS

- Ketamine dose-dependently increases TrkB signaling in the cortex.
- Sedative-anesthetic doses of 6,6-*d*₂-ketamine increase TrkB phosphorylation.
- Slow EEG oscillations correlate with ketamine-induced TrkB signaling.
- *cis*-HNK produces negligible acute effects on slow oscillations and TrkB signaling.

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ABSTRACT

Subanesthetic rather than anesthetic doses are thought to bring the rapid antidepressant effects of the NMDAR (*N*-methyl-D-aspartate receptor) antagonist ketamine. Among molecular mechanisms, activation of BDNF receptor TrkB along with the inhibition of GSK3 β (glycogen synthase kinase 3 β) are considered as critical molecular level determinants for ketamine's antidepressant effects. Hydroxynorketamines (2*R*,6*R*)-HNK and (2*S*,6*S*)-HNK, non-anesthetic metabolites of ketamine, have been proposed to govern the therapeutic effects of ketamine through a mechanism not involving NMDARs. However, we have shown that nitrous oxide, another NMDAR blocking anesthetic and a putative rapid-acting antidepressant, evokes TrkB-GSK3 β signaling alterations during rebound slow EEG (electroencephalogram) oscillations. We investigated here the acute effects of ketamine, 6,6-*d*₂-ketamine (a ketamine analogue resistant to metabolism) and *cis*-HNK that contains (2*R*,6*R*) and (2*S*,6*S*) enantiomers in 1:1 ratio, on TrkB-GSK3 β signaling and concomitant electroencephalographic (EEG) alterations in the adult mouse cortex. Ketamine dose-dependently increased slow oscillations and phosphorylations of TrkB^{Y816} and GSK3 β ^{S9} in crude brain homogenates (i.e. sedative/anesthetic doses (> 50 mg/kg, i.p.) produced more prominent effects than a subanesthetic dose (10 mg/kg, i.p.)). Similar, albeit less obvious, effects were seen in crude synaptosomes. A sedative dose of 6,6-*d*₂-ketamine (100 mg/kg, i.p.) recapitulated the effects of ketamine on TrkB and GSK3 β phosphorylation while *cis*-HNK at a dose of 20 mg/kg produced negligible acute effects on TrkB-GSK3 β signaling or slow oscillations. These findings suggest that the acute effects of ketamine on TrkB-GSK3 β signaling are by no means restricted to subanesthetic (i.e. antidepressant) doses and that *cis*-HNK is not responsible for these effects.

Abbreviations: BDNF, brain-derived neurotrophic factor; CREB, cAMP related element binding protein; ECT, electroconvulsive therapy; EEG, electroencephalogram; EMG, electromyogram; GSK3 β , glycogen synthase kinase 3 β ; TrkB, tropomyosin related kinase B

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1. Introduction

Ketamine has been in active clinical use in anesthesiology for over half a century, but recently new interest has sparked from its ability to rapidly and efficiently ameliorate depressive symptoms in treatment resistant patients (Berman et al., 2000). The dissociative anesthesia produced by ketamine is different from many other sedatives and anesthetics, since it is not a hypnotic drug (Sinner and Graf, 2011). Unlike most general anesthetics, ketamine-induced blockade of the NMDARs (*N*-methyl-D-aspartate receptors) leads to the emergence of dissociative states, where patients may experience being conscious while being drawn away from their sensory perception (Mathew and Zarate, 2016). The level of dissociation is increased with dose, with higher doses deepening into hallucinatory-like states of open and closed-eye visuals and extreme perturbations of thought and bodily sensation (Garfield et al., 1972). Ketamine dose-dependently regulates the emergence of slow EEG oscillations (delta, ~1–4 Hz), which coincide with the deepening of anesthesia, while lower subanesthetic doses preferentially influence high frequency gamma oscillations (25–100 Hz) (Hiyoshi et al., 2014; Maksimow et al., 2006; Purdon et al., 2015; Zanos et al., 2016). Increased gamma oscillations are thought to be connected to the acute psychotomimetic and excitatory effects of ketamine on cortical neurons (Purdon et al., 2015). The effects of ketamine on delta oscillations and EEG slowing in general are less pronounced than seen with general anesthetics and sedatives acting on GABA_A channels or α_2 -adrenergic receptors (Kohtala et al., 2019; Maksimow et al., 2006; Purdon et al., 2015).

Subanesthetic doses of ketamine (0.5 mg/kg, over a 40-min i.v. infusion) have been consistently used in clinical research and have repeatedly demonstrated efficacy in ameliorating depressive symptoms. Intriguingly, some studies have reported a relationship between the antidepressant response and the psychotomimetic effects or dissociative symptoms produced by the higher-end of subanesthetic doses of ketamine (Lai et al., 2014; Loo et al., 2016; Luckenbaugh et al., 2014; Niciu et al., 2018; Sos et al., 2013; Xu et al., 2016). Generally, higher anesthetic doses of ketamine are not however thought to elicit antidepressant effects, but doses up to 1 mg/kg have been demonstrated to be effective (Fava et al., 2018). Yet, knowledge of the dose-dependence of ketamine's effects in both clinical and preclinical settings is incomplete.

A large number of preclinical studies have elucidated the neuropharmacological mechanisms behind ketamine's rapid-acting antidepressant effects. Subanesthetic doses have been proposed to increase glutamate release either via the preferential blockade of NMDARs on GABAergic interneurons (Homayoun and Moghaddam, 2007; Moghaddam et al., 1997) or through direct antagonism of extrasynaptic NMDARs on pyramidal neurons, resulting in compensatory increases of excitatory drive in the prefrontal cortex (Miller et al., 2016). Indeed, low doses of ketamine have been reported to increase glutamate activity as measured by microdialysis in rats, while anesthetic doses lead to decreases in glutamate release (Moghaddam et al., 1997). Furthermore, this surge in glutamate after subanesthetic ketamine administration has been proposed to underlie the rapid-acting antidepressant effects via the regulation of AMPARs (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors), since blockade of these channels with NBQX abolishes ketamine's antidepressant like behavioral responses in mice and rats (Koike et al., 2011; Koike and Chaki, 2014; Maeng et al., 2008). These events have been proposed to further lead into the activation of BDNF (brain-derived neurotrophic factor) receptor TrkB, mTOR (mammalian target of rapamycin) and p44/42-MAPK (mitogen-activated protein kinase) signaling along with the inhibition of GSK3 β (glycogen synthase kinase 3 β), which have been intimately connected with ketamines' antidepressant effects in molecular studies and behavioral models (Beurel et al., 2011; Li et al., 2010; Réus et al., 2014).

Recently an animal study investigating the effects of ketamine's

metabolites suggested that particular metabolites, hydroxynorketamines (HNKs), are responsible for ketamine's rapid antidepressant effects (Zanos et al., 2016). Both (2*R*,6*R*) and (2*S*,6*S*) enantiomers of HNK produce antidepressant-like effects in mice, although (2*R*,6*R*) is more effective (Zanos et al., 2016). These effects of HNK are suggested to be produced through a more direct modulation of AMPAR activity without the involvement of NMDARs (Zanos et al., 2016). This hypothesis, however, contrasts with many studies emphasizing the critical role of NMDAR antagonism as the main mechanism of ketamine's action, and has subsequently raised important debate about the neuropharmacological mechanisms involved (Collingridge et al., 2017; Suzuki et al., 2017).

Electroconvulsive therapy (ECT) is still among the most effective antidepressant treatments in clinical use. The ability of ECT to ameliorate depressive symptoms has been associated with post-ictal (i.e. after seizure) emergence of slow EEG oscillations and/or burst suppression pattern rapidly after convulsions, but also the general slowing of the EEG during the course of the treatments (Fink and Kahn, 1957; Folkerts, 1996; Nobler et al., 1993; Perera et al., 2004; Sackeim et al., 1996; Suppes et al., 1996). These effects of ECT on EEG encouraged scientists already decades ago to test whether the silencing of cortical activity induced by general anesthetics might be sufficient enough to elicit antidepressant responses. Some pilot clinical studies of isoflurane and propofol for depression have indeed shown promise (Engelhardt et al., 1993; Langer et al., 1995, 1985; Mickey et al., 2018; Weeks et al., 2013). We have previously demonstrated that brief burst-suppressing isoflurane anesthesia essentially regulates key molecular alterations implicated in antidepressant mechanisms and produces antidepressant-like behavioral outcomes in mice (Antila et al., 2017; Theilmann et al., 2019). Similar phenotypic effects of isoflurane have been later observed in rats by others (Brown et al., 2018). Most intriguingly, our latest study demonstrated that subanesthetic nitrous oxide (N₂O), another NMDAR antagonist and a putative rapid-acting antidepressant (Nagele et al., 2015), produces "rebound" cortical slow EEG oscillations after the acute pharmacological effects have subsided along with the regulation of TrkB-GSK3 β signaling (Kohtala et al., 2019).

In the present study we investigated the dose-dependent effects of ketamine on some of the molecular pathways heavily implicated in its rapid-antidepressant action along with quantitative EEG to recapitulate changes on cortical brain activity. Moreover, we used 6,6-dideuteroketamine (a ketamine analogue resistant to metabolism) along with *cis*-HNK that contains (2*R*,6*R*) and (2*S*,6*S*) enantiomers in 1:1 ratio to gain further insight into the mechanistic basis of such molecular alterations regulated by ketamine.

2. Methods and materials

2.1. Animals

Adult male C57BL/6JRCcHsd mice (Harlan Laboratories, Venray, The Netherlands) were used. Animals were maintained in the animal facility of University of Helsinki (Finland) under standard conditions (21 °C, 12-h light-dark cycle) with free access to food and water. The animal experiments were carried out according to the guidelines of the Society for Neuroscience and were approved by the County Administrative Board of Southern Finland (License: ESAVI/10527/04.10.07/2014).

2.2. EEG recordings and data analysis

For the implantation of electrodes, mice were anesthetized with isoflurane (3% induction, 1.5–2% maintenance). Lidocaine (10 mg/mL) was used as local anesthetic and buprenorphine (0.1 mg/kg, s.c.) for postoperative care. Two epidural screw EEG (electroencephalogram) electrodes were placed above the fronto-parietal cortex. A further screw served as mounting support. Two silver wire electrodes were implanted

in the nuchal muscles to monitor the EMG (electromyogram). After the surgery, mice were single-housed in Plexiglas boxes. After a recovery period of 5–7 days, animals were connected to flexible counterbalanced cables for EEG/EMG recording and habituated to recording cables for three days.

Baseline EEG (10–15 min) recordings of awake animals were conducted prior i.p. injection of saline or test compound. All recordings were performed during the light period.

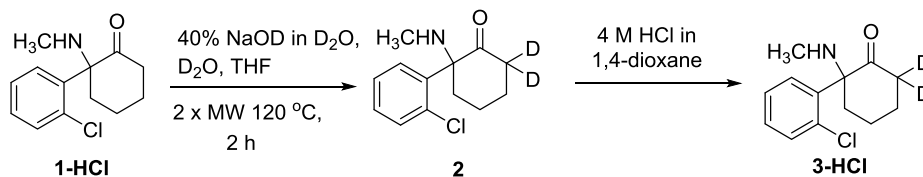
The EEG and EMG signals were amplified (gain 5 or 10 K) and filtered (high pass: 0.3 Hz; low pass 100 Hz; notch filter) with a 16-channel AC amplifier (A-M System, model 3500), sampled at 254 Hz or 70 Hz with 1401 unit (CED), and recorded using Spike2 (version 8.07, Cambridge Electronic Devices). The processing of the EEG data was obtained using Spike2 (version 8.07, Cambridge Electronic Devices). EEG power spectra were calculated within the 1–50 Hz frequency range by fast Fourier transform (FFT = 256, Hanning window, 1.0 Hz resolution). Oscillation power in each bandwidth ($\Delta = 1$ –4 Hz; $\theta = 4$ –7 Hz; $\alpha = 7$ –12 Hz; $\beta = 12$ –25 Hz; γ low = 25–40 Hz; γ high = 60–100 Hz) was computed in 30–300-sec epochs from spectrograms (FFT size: 1024 points) for each animal. Representative sonograms were computed using a Hanning window with a block size of 512.

2.3. Pharmacological treatments

All drug treatments were done during the light phase between 9:00–12:00 a.m. Ketamine-HCl (10–200 mg/kg; Ketaminol®, Intervet International B.V.), 6,6- d_2 -ketamine-HCl (see below; 100 mg/kg) and *cis*-6-hydroxynorketamine-HCl (10–20 mg/kg; Tocris) were diluted in isotonic saline solution and injected intraperitoneally with an injection volume of 10 mL/kg.

2.4. Synthesis of 6,6- d_2 -ketamine-HCl

Reagents and solvents for the syntheses were purchased from Sigma-Aldrich (Darmstadt, Germany) and VWR International Oy (Helsinki, Finland). Racemic ketamine hydrochloride was purchased from YA Apteekki (Helsinki, Finland). ^1H NMR and ^{13}C NMR spectra in CDCl_3 or CD_3OD at ambient temperature were recorded on a Bruker Avance 400 MHz NMR (Bruker Corporation, Billerica, MA, USA) with the smart probe. Chemical shifts (δ) are given in parts per million (ppm) relative to the NMR reference solvent signals (CDCl_3 : 7.26 ppm; CD_3OD : 3.31 ppm for ^1H NMR and CDCl_3 : 77.16 ppm; CD_3OD : 49.0 ppm for ^{13}C NMR). Multiplicities are indicated by s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), t (triplet), m (multiplet). The coupling constants J are quoted in Hertz (Hz). HRMS spectra were recorded using Waters Acquity UPLC®-system (Waters, Milford MA, USA) with Acquity UPLC® BEH C18 column (1.7 μm , 50 mm \times 2.1 mm, Waters, Wexford, Ireland) with Waters Synapt G2 HDMS (Waters, Milford MA, USA) with the ESI (+), high resolution mode. The mobile phase consisted of H_2O (A) and acetonitrile (B) both containing 0.1% HCOOH . Microwave syntheses were performed in sealed tubes using Biotage Initiator + instrument (Uppsala, Sweden) equipped with an external IR sensor.



2.4.1. Synthesis of 6,6- d_2 -ketamine-HCl 3 (1)

Racemic ketamine-HCl 1 (0.60 g, 2.2 mmol (YA Apteekki, Helsinki, Finland) was dissolved in dry tetrahydrofuran (6 mL) and D_2O

(2.25 mL). To this solution a 40% solution of NaOD in D_2O (2.25 mL) was added. The sealed tube was microwave-irradiated at 120 °C for 2 h. The resulting mixture was poured to 1 M aqueous solution of HCl (20 mL). The white precipitate was filtered and washed with water and dried to yield 2 (394 mg, 75%). ^1H NMR and MS spectra showed that the isotopic purity was not over 90%, so the above procedure was repeated for 2 (379 mg) using a 40% solution of NaOD in D_2O (1 mL). 6,6- d_2 -Ketamine 2 was obtained as a white solid (342 mg, 90%). ^1H NMR (400 MHz, CDCl_3) δ 7.55 (dd, $J = 7.9$, 1.7 Hz, 1H), 7.38 (dd, $J = 7.8$, 1.5 Hz, 1H), 7.32 (ddd, $J = 7.8$, 7.3, 1.5 Hz, 1H), 7.27–7.21 (m, 1H), 2.84–2.73 (m, 1H), 2.15 (bs, 1H), 2.10 (s, 3H), 2.03–1.95 (m, 1H), 1.90–1.80 (m, 1H), 1.74 (m, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 209.5, 137.9, 134.0, 131.4, 129.6, 128.8, 126.8, 70.4, 39.1 (m), 38.8, 29.3, 28.2, 22.0.

6,6- d_2 -Ketamine 2 (0.30 g) was dissolved in dry 1,4-dioxane (5 mL) and a 4 M solution of HCl in 1,4-dioxane (1 mL) was added. The formed white precipitate was filtered and dried to yielding 6,6- d_2 -ketamine-HCl 3 (0.26 g, 75%). 3 was dissolved to water, filtered and dried prior the use.

^1H NMR (400 MHz, CD_3OD) δ 7.94–7.90 (m, 1H), 7.68–7.58 (m, 3H), 3.42–3.34 (m, 1H), 2.39 (s, 3H), 2.17–2.10 (m, 1H), 1.96–1.84 (m, 2H), 1.83–1.68 (m, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ 208.4, 135.9, 133.9, 133.4, 133.2, 129.7, 129.2, 73.6, 40.3 (m), 37.4, 30.9, 27.9, 22.8. ^1H NMR and ^{13}C NMR were in agreement with reported data (Zanos et al., 2016). HRMS (ESI⁺): Calcd for $\text{C}_{13}\text{H}_{15}\text{D}_2\text{ClNO}^+$ 240.1124; found 240.1130.

2.5. Dissection and processing of brain samples

Animals were euthanized at indicated times after the treatments by rapid cervical dislocation followed by decapitation (Kohtala et al., 2016). Bilateral medial prefrontal cortex (including prelimbic and infralimbic cortices) was rapidly dissected on a cooled dish and stored at -80°C until further processing (Rantamäki et al., 2007). For the analysis of crude brain homogenates the samples were homogenized in lysis buffer (137 mM NaCl, 20 mM Tris, 1% NP-40, 10% glycerol, 48 mM NaF, H_2O , Complete inhibitor mix (Roche), PhosStop (Roche)). After ~ 15 -min incubation on ice, samples were centrifuged (16000g, 15 min, 4°C) and the resulting supernatant collected for Western blot analysis. For the preparation of crude synaptosomes, brain samples were homogenized in 10% (w/v) ice-cold buffer containing 0.32 M sucrose, 20 mM HEPES pH 7.4, 1 mM EDTA, 1 X protease inhibitor cocktail (Roche, Mannheim, Germany), 5 mM NaF, 1 mM Na_3VO_4 , and 5 mM Na_2HPO_4 . After centrifugation of the homogenate at 2800 rpm for 10 min at 4°C , the supernatant was transferred to a new tube and centrifuged at 12 000 rpm for 10 min. The supernatant (cytosolic fraction) was removed and the resultant pellet, designated the crude synaptosomal fraction, was resuspended in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 1 X protease inhibitor cocktail, 2 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4 , and 5 mM Na_2HPO_4).

2.6. Western blotting

Samples (20–50 μg protein) were separated with SDS-PAGE under reducing and denaturing conditions and blotted to a PVDF (poly-

vinylidene difluoride) or nitrocellulose membrane using standard protocols. After blocking, the membranes were incubated with the following primary antibodies: *anti*-p-TrkB^{Y816} (#4168; 1:250–1000, Cell

signaling technology (CST)), anti-TrkB (#4603; 1:1000, CST or, #AF1494; 1:500, #R&D Systems), pan-Trk (sc-11; 1:1000; Santa Cruz Biotechnology (SCB)), anti-p-CREB (#9191S; 1:1000, CST), anti-p-p70S6K^{T421/S424} (#9204S; 1:500–1000, CST), anti-p-GSK3^{S9} (#9336; 1:1000, CST or #5558; 1:500, CST), anti-p-p44/42-MAPK^{Thr202/Y204} (#9106; 1:1000, CST), anti-GSK3^β (#9315; 1:1000, CST or #9832; 1:1000, CST), anti-p70S6K (#2708; 1:1000, CST), anti-p44/42-MAPK (#9102; 1:1000, CST), anti-GAPDH (#2118; 1:10 000, CST), anti- β -actin (926–42212; 1:3000, LI-COR), anti-Synaptotagmin 1 (ANR-003; 1:400, Alomone), anti-BDNF (ab108319; 1:1000, Abcam), anti-NMDAR2B (#14544; 1:1000, CST), and anti-PSD95 (#2507; 1:500, CST). Further, the membranes were washed with TBS/0.1% Tween (TBST) and incubated with horseradish peroxidase conjugated secondary antibodies (1:10000, 1 h at room temperature; Bio-Rad) or appropriate IRDye conjugated secondary antibody (1:15000, 1 h at room temperature; LI-COR): IRDye 800CW goat anti-rabbit IgG, IRDye 680RD goat anti-rabbit IgG, IRDye 800CW goat anti-mouse IgG, IRDye 680RD goat anti-mouse IgG, IRDye 800CW donkey anti-goat IgG, and IRDye 680RD donkey anti-rabbit IgG. After subsequent washes, secondary antibodies were visualized using enhanced chemiluminescence (ECL Plus, ThermoScientific) for detection by Biorad ChemiDoc MP camera (Bio-Rad Laboratories) or infrared signals using the Odyssey CLx infrared imaging system. ImageJ and Image Studio software Version 5.0 (LI-COR) were used for quantifications of chemiluminescence and fluorescence blots, respectively.

2.7. Statistical analyses

Depending on whether data were normally distributed or not, either parametric or nonparametric tests were used for statistical evaluation. In case of more than two groups, analysis of variance (ANOVA) with appropriate *post hoc* test was used. All statistical analyses were performed with the Prism 7 software from GraphPad (La Jolla, CA, USA). All tests were used two-sided; a $P \leq 0.05$ was considered significant. For statistical analyses and n numbers see [Supplementary Table 1](#).

3. Results

3.1. Ketamine regulates TrkB-GSK3 β signaling dose-dependently during increased slow oscillations

Subanesthetic dose of ketamine (10 mg/kg, i.p.) produced a modest increase in gamma high oscillations (60–100 Hz), suggestive of the acute psychopharmacological effects, while effects on other frequency bands were limited ([Fig. 1A–B](#)). A higher dose of ketamine (100 mg/kg, i.p.), however, produced a rapid and significant increase in delta-frequency (1–4 Hz) power along with increases in theta (4–7 Hz), beta (12–25 Hz), gamma low (25–40 Hz) and gamma high power (60–100 Hz), while decreasing alpha frequency power (7–12 Hz). Overall, these observations are well in line with previous findings ([Hiyoshi et al., 2014; Zanos et al., 2016](#)) and further demonstrate the dose-dependent effects of ketamine on cortical activity measured by EEG.

We next investigated the dose-dependent effects of ketamine on TrkB and GSK3 β phosphorylation in the medial prefrontal cortex (mPFC). We initially focused our studies to crude homogenates since various antidepressants have been shown to regulate TrkB signaling in such preparations ([Antila et al., 2017; Aonurm-Helm et al., 2015; Autry et al., 2011; Di Lieto et al., 2012; Dong et al., 2017; Kohtala et al., 2019; Rantamäki et al., 2007, 2006; Saarelainen et al., 2003; Sun et al., 2016](#)). We saw significant increases in TrkB^{Y816}, p70S6K^{T421/S424} (downstream of mTOR) and GSK3 β ^{S9} phosphorylation 30 min after a high dose (100 mg/kg, i.p.) of ketamine, while a subanesthetic dose (10 mg/kg, i.p.) failed to produce any changes ([Fig. 1C](#)). Similar, albeit less pronounced, effects were seen when the brain samples were collected 15 min post-injection ([Fig. S1](#)).

We next investigated the effects of ketamine on TrkB, p70S6k and GSK3 β phosphorylations in synaptosomal preparations ([Fig. S2](#)), which have also been used to assess antidepressant-induced changes on these molecular events. While phosphorylation of TrkB^{Y816} remained unaffected, phosphorylation of GSK3 β ^{S9} was increased by an anesthetic dose of ketamine, and phosphorylation of p70S6k^{T421/S424} was upregulated by both subanesthetic and high dose 30 min post-injection ([Fig. S3](#)). Notably, no clear dose-dependent effects of ketamine were seen in these analyses ([Fig. S3](#)).

To confirm the dose-dependent effects of ketamine on these molecular effects, we performed another study with subanesthetic (10 mg/kg, i.p.), sedative (50 mg/kg, i.p.) and anesthetic (200 mg/kg, i.p.) doses of ketamine. Of selected doses only the highest produced obvious effects on TrkB^{Y816}, p70S6k^{T421/S424} and GSK3 β ^{S9} phosphorylation in crude brain homogenates 30 min post-injection ([Fig. 2](#)). Since the effects of ketamine on these phosphorylation changes were readily detected in crude homogenates, such preparations were also used in subsequent studies.

3.2. Regulation of TrkB-GSK3 β signaling by ketamine is independent of hydroxynorketamines

To investigate whether HNK regulates TrkB and GSK3 β signaling, we subjected mice to acute treatments with *cis*-HNK that contains (2R,6R) and (2S,6S) enantiomers in 1:1 ratio and collected tissue samples 30 min later from the mPFC. The phosphorylation of TrkB^{Y816}, p70S6k^{T421/S424} and GSK3 β ^{S9} remained unaltered after an acute treatment of *cis*-HNK (20 mg/kg, i.p.) ([Fig. 3A](#)). HNK produced no clear acute EEG alterations compared to saline treated mice ([Fig. 3B–C](#)).

High doses (> 100 mg/kg, i.p.) of ketamine bring sedation within few minutes, along with increased slow oscillations in mice ([Fig. 1](#)). Indeed, an intraperitoneal dose of 200 mg/kg ketamine induced pTrkB, p70S6k and pGSK3 β levels in the mouse mPFC already within 3 min when its metabolism into HNK is likely marginal ([Fig. 4A](#)). To further investigate whether the metabolism of ketamine to HNK is involved in ketamine-induced regulation of TrkB-GSK3 β signaling, we deuterated ketamine at the C6 position (6,6-*d*₂-ketamine) to hinder its metabolism and compared this ketamine analogue to the acute effects of ketamine. Importantly, a high dose of 6,6-*d*₂-ketamine (100 mg/kg, i.p.) essentially recapitulated the phosphorylation effects of an equivalent dose of ketamine on TrkB, p70S6K and GSK3 β ([Fig. 4B](#)).

4. Discussion

Activation of BDNF receptor TrkB and inhibition of GSK3 β have been intimately associated with the mechanism of action of rapid-acting antidepressant ketamine ([Duman and Aghajanian, 2012; Rantamäki and Yalcin, 2016](#)). We have recently shown that nitrous oxide, another NMDAR antagonist and a putative rapid-acting antidepressant ([Nagele et al., 2015](#)), acutely upregulates markers of cortical excitability, followed by a period of rebound slow EEG oscillations during which TrkB-GSK3 β alterations become evident ([Kohtala et al., 2019](#)). In addition, we have previously shown that isoflurane anesthesia, at a dosing regimen shown to bring rapid antidepressant effects in a subset of patients ([Langer et al., 1995](#)), targets these very same signaling mechanisms ([Antila et al., 2017; Theilmann et al., 2019](#)). The rationale to investigate isoflurane in clinical trials was based upon the idea that post-ictal (i.e. after seizure) emergence of burst-suppression and/or electrocortical silence predicts the onset-of-action of ECT ([Krystal et al., 1995; Langer et al., 1995, 1985; Nobler et al., 1993](#)). In contrast to ECT, isoflurane reliably and reproducibly produces EEG burst suppression with adequate dosing. Notably, electroconvulsive shock (an animal model of ECT) has not been shown to rapidly activate TrkB ([Hansen et al., 2007](#)) although it readily increases BDNF synthesis ([Nibuya et al., 1995](#)). ECS and volatile convulsant flurothyl does, however, increase GSK3 β ^{S9} and TrkB^{Y816} phosphorylation gradually after seizure

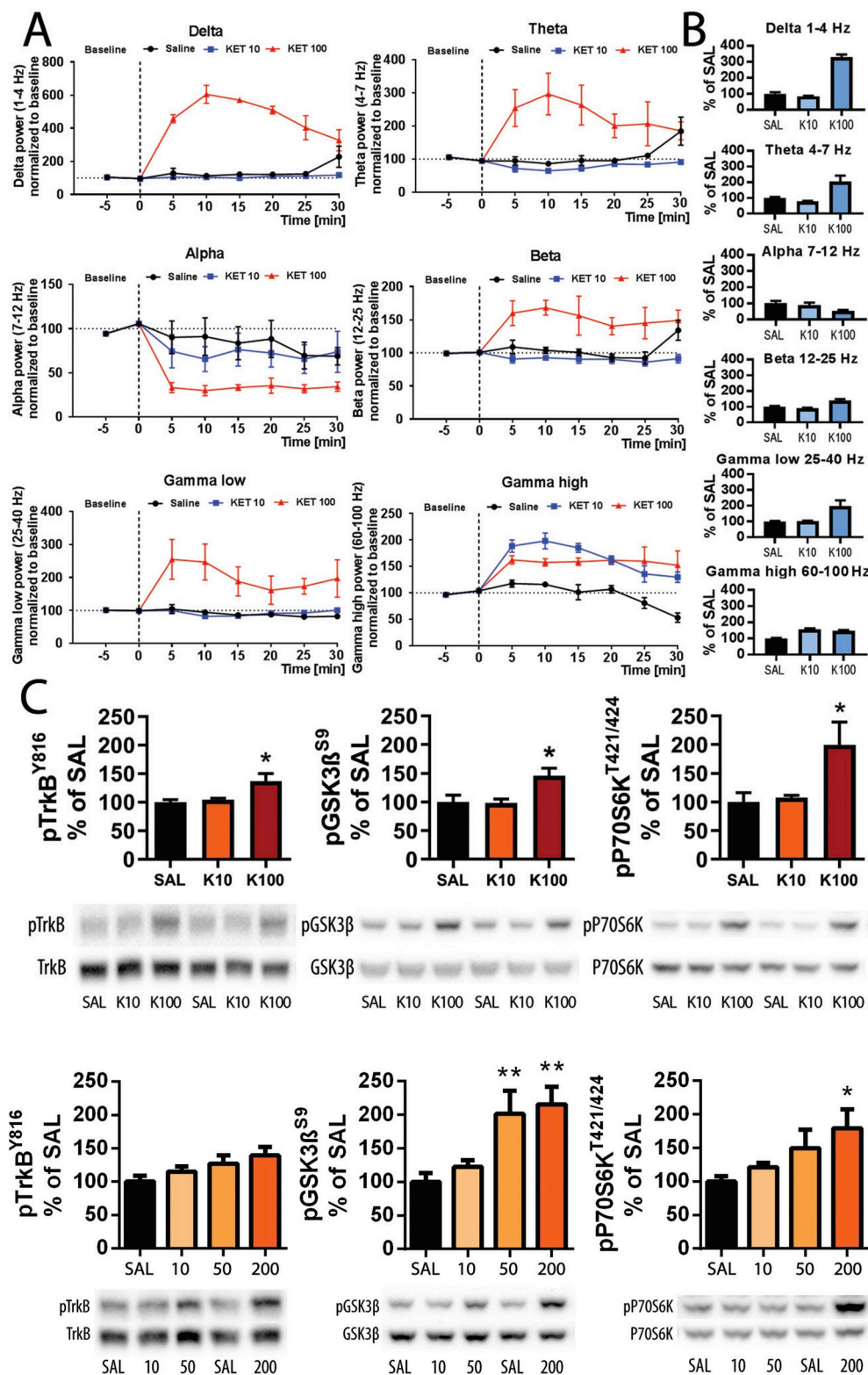


Fig. 1. (A) Normalized power of major EEG oscillations during ketamine (10 and 100 mg/kg) (data analyzed in 5 min bins). Dashed vertical line indicates injection point (0 min). (B) Major EEG oscillation frequency band power of ketamine treatments represented as area under curve (AUC) from 30 min of recording. (C) Phosphorylation of TrkB^{Y816}, GSK3β^{S9} and p70S6k^{T421/424} in the adult mouse medial prefrontal cortex 30 min after an i.p. injection of saline (SAL) and ketamine (K10, 10 mg/kg; K100, 100 mg/kg). Approximate molecular weight (MW) for each protein band of interest is given in kilodaltons (kDa). Data are means ± S.E.M. * < 0.05, ** < 0.01, *** < 0.005 (for statistical analyses and *n* numbers see [Supplementary Table 1](#)).

termination (Basar et al., 2013; Kang et al., 2004; Kohtala et al., 2019; Roh et al., 2003). These changes co-incide with marked slowing of EEG (Kohtala et al., 2019).

The present study demonstrates that ketamine regulates TrkB-GSK3β signaling in a dose-dependent manner in the mouse prefrontal cortex. A low subanesthetic dose of ketamine (10 mg/kg, i.p.) produced negligible acute changes in phosphorylation while it facilitated a modest increase in gamma high oscillations (60–100 Hz). A higher

subanesthetic dose (100 mg/kg, i.p.) produced a significant increase in TrkB^{Y816}, p70S6k^{T421/424} and GSK3β^{S9} while facilitating the power of delta-frequency (1–4 Hz) oscillations. Increases in theta (4–7 Hz), beta (12–25 Hz), gamma low (25–40 Hz) and gamma high (60–100 Hz) along with decreases in alpha (7–12 Hz) frequency power were also observed with the higher dose. Dose-dependent effects of ketamine on TrkB-GSK3β phosphorylation was confirmed in a follow up experiment, wherein most prominent effects were seen with 200 mg/kg of ketamine

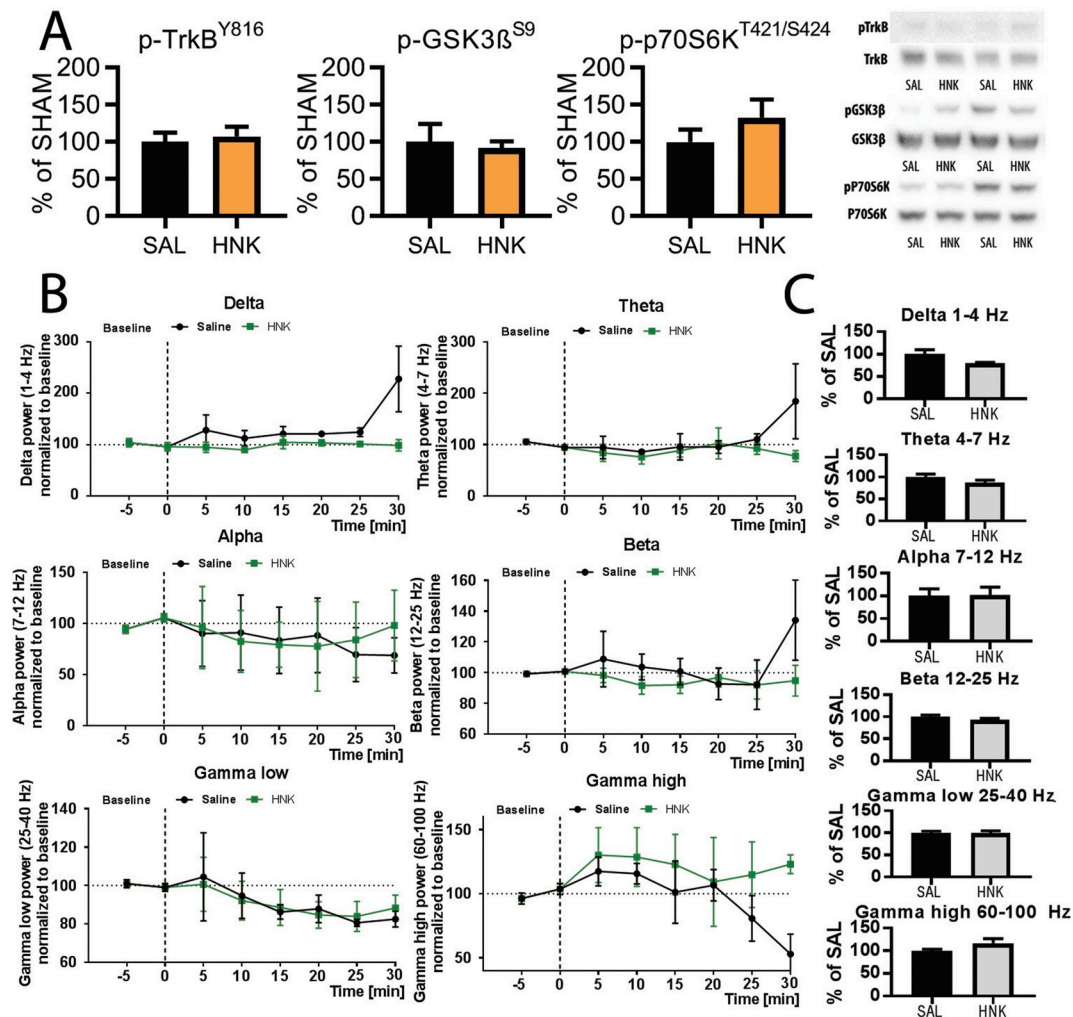


Fig. 3. (A) Phosphorylation of TrkB^{Y816}, GSK3β^{S9} and p70S6K^{T421/S424} in the adult mouse medial prefrontal cortex 30 min after an i.p. injection of saline (SAL) and *cis*-6-hydroxynorketamine (HNK, 20 mg/kg). (B) Normalized power of major EEG oscillations during HNK (20 mg/kg) (data analyzed in 5 min bins). Dashed vertical line indicates injection point (0 min). (C) Major EEG oscillation frequency band power of HNK treatment represented as area under curve (AUC) from 30 min of recording. Data are means \pm S.E.M. * < 0.05 , ** < 0.01 , *** < 0.005 (for statistical analyses and *n* numbers see [Supplementary Table 1](#)).

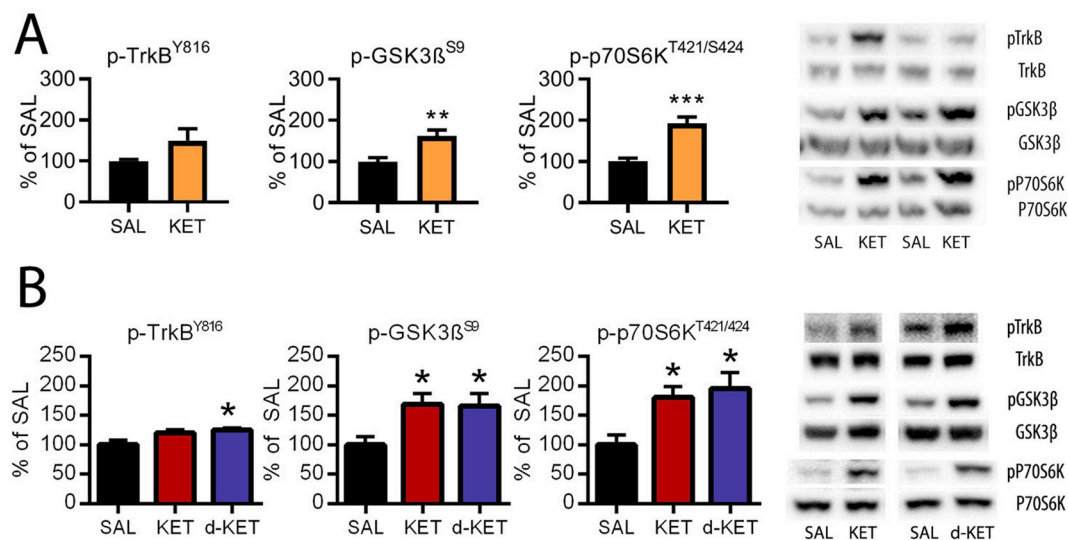


Fig. 4. (A) Ketamine produces increases in TrkB^{Y816}, GSK3β^{S9} and p70S6K^{T421/S424} in the adult mouse medial prefrontal cortex 3 min after an injection of saline (SAL) or ketamine (KET, 200 mg/kg). (B) Effects of KET (100 mg/kg, i.p.; 30 min) and 6,6-dideuteroketamine (d-KET, 100 mg/kg, i.p.; 30 min) on p-TrkB^{Y816}, p-GSK3β^{S9} and p-p70S6K^{T421/S424}. Data are means \pm S.E.M. * < 0.05 , ** < 0.01 , *** < 0.005 (for statistical analyses and *n* numbers see [Supplementary Table 1](#)).

(i.p.). Thus, in our hands sedative/anesthetic doses of ketamine preferentially regulate TrkB-GSK3 β signaling in the adult mouse brain, at least within the selected time-frame (Lindholm et al., 2012).

One of the goals of our study was to investigate whether HNKs play a role in the ability of ketamine to regulate TrkB and GSK3 β signaling in the brain. A recent study indicates that (2R,6R)-HNK, at a dose of 30 mg/kg, increases phosphorylation of mTOR (a kinase downstream of TrkB and upstream of p70S6k, respectively) in the mouse medial prefrontal cortex (Fukumoto et al., 2019). The authors did not, however, investigate TrkB and GSK3 β phosphorylation or the effects of HNK at a dose more comparable with 10 mg/kg of ketamine (Fukumoto et al., 2019). The antidepressant effects of (2R,6R)-HNK emerge at ~5–10 mg/kg i.p. while > 25 mg/kg i.p. is needed for the (2S,6S) enantiomer (Zanos et al., 2016). We thus selected a dose of *cis*-HNK that contain 10 mg/kg (2R,6R)-HNK (i.e. 20 mg/kg, i.p.), a dose consistently used by Zanos et al. and others. This dose produced negligible acute effects on TrkB and GSK3 β phosphorylation. Furthermore, we demonstrate that a sedative dose of a deuterated ketamine analogue (resistant to metabolism) produces essentially similar effects on TrkB and GSK3 β phosphorylation as the equivalent dose of ketamine. The independence of these effects from ketamine's HNK metabolites is also supported by altered phosphorylation levels evident already after 3 min of an anesthetic dose of ketamine. Taken together, these findings strongly suggest that the effects of ketamine on TrkB-GSK3 β signaling are by no means restricted to subanesthetic doses and that *cis*-HNK is not responsible for these effects. In contrast, the highest sedative doses produced most obvious acute effects, which also concomitantly increased slow EEG oscillations. We cannot, however, rule out the possibility that HNK or its specific enantiomers, at some other doses or at different time points, regulate TrkB-GSK3 β phosphorylation. (2R,6R)-HNK does not, however, produce sedation even at very high doses in mice (Zanos et al., 2016).

The preferential ability of high doses of ketamine to regulate TrkB-GSK3 β signaling alterations may be related to the pharmacologically induced brain state, evidenced by the emergence of slow oscillations that are characteristic physiological features of deep sleep. It is thus plausible that the phosphorylation events set forth by ketamine are representative not only of a primary pharmacological target of the drug and its relevant cascade of downstream effectors, but a more generally altered brain state. This state has striking similarities with post-ictal state and sedation, and could be the result of pronounced changes in excitatory and/or inhibitory drive, energy metabolism, or a number of other hypothetical mechanisms yet to be investigated. The ability of isoflurane anesthesia (Antila et al., 2017) and medetomidine-induced sedation (Kohtala et al., 2019) to readily and rapidly regulate TrkB-GSK3 β signaling is strictly in line with such a hypothesis.

Based on our previous findings we have proposed a hypothesis of the two-phases of action of rapid-acting antidepressant treatments (Kohtala et al., 2019). Essentially, we suggest that rapid-acting treatments increase cortical excitability during their acute pharmacological effects, which is followed by a homeostatic rebound increase of slow EEG oscillations, during which the regulation of TrkB-GSK3 β signaling is also increased. While the role of slow EEG oscillations and mechanisms of sleep in the action of rapid-acting antidepressants remains debated, several interesting perspectives have emerged. For example, slow oscillations during subsequent night after ketamine treatment have been found to correlate with the antidepressant efficacy (Duncan et al., 2013), and ketamine has also been proposed to induce changes on circadian systems (Duncan et al., 2017; Orozco-Solis et al., 2017). Furthermore, we propose that the direct facilitation of slow EEG oscillations and TrkB-GSK3 β signaling is not sufficient for rapid-antidepressant responses without the preceding cortical excitation and the regulation of related plasticity-related immediate early genes (Kohtala et al., 2019). Thus, the ability of higher doses of ketamine to more prominently regulate these signaling pathways may not be directly related to the antidepressant-like behavioral effects or clinically

meaningful antidepressant responses. Indeed, anesthetic doses of ketamine (> 1 mg/kg, i.v.) are not considered effective against major depression and do not accelerate or improve therapeutic effects of ECT (Carspecken et al., 2018; Järventausta et al., 2013; Rasmussen et al., 2014) (see however Okamoto et al., 2010). Moreover, if anything, antidepressant doses of ketamine (0.25–0.5 mg/kg, i.v.) seem to acutely dampen EEG oscillations within delta-theta range in both humans and rodents (de la Salle et al., 2016; Hong et al., 2010; Kohtala et al., 2019; Muthukumaraswamy et al., 2015; Shadli et al., 2018). Importantly however, these effects may be time-dependent as gradual emergence of slow EEG oscillations have been noted in animals after the acute pharmacological effects of low-dose of ketamine have dissipated (Feinberg and Campbell, 1995, 1993; Kohtala et al., 2019). That said, the sequence of the appearance of these EEG alterations and molecular signaling events, rather than the existence of these as such, may be important for rapid antidepressant effects (Kohtala et al., 2019). Further work in both preclinical and clinical settings is warranted to unveil the complex dose- and time-dependent molecular and concomitant electrophysiological alterations behind ketamine's (and its metabolites) antidepressant effects.

5. Conclusions

Our results suggest that ketamine regulates TrkB and GSK3 β signaling pathways dose-dependently and independently from its HNK metabolites. These findings further deepen the knowledge of ketamine's effects on these molecular signaling pathways and strengthen an intriguing correlation between the regulation of key molecular pathways implicated in rapid antidepressant effects and the pharmacologically induced brain state characterized by slow oscillations and sedation, while raising important questions about the nature of these signaling alterations in antidepressant responses altogether.

Disclosure

University of Helsinki has filed a patent application wherein data presented in this manuscript have been disclosed (S.K., W.T. and T.R. as inventors).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neuropharm.2019.107684>.

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